Evidence for a role of sterol 27-hydroxylase in glucocorticoid metabolism in vivo

Vögeli, Isabelle ; Jung, Hans H ; Dick, Bernhard ; Erickson, Sandra K ; Escher, Robert ; Funder, John W ; Frey, Felix J ; Escher, Geneviève

Abstract: The intracellular availability of glucocorticoids is regulated by the enzymes 11 -hydroxysteroid dehydrogenase 1 (HSD11B1) and 11 -hydroxysteroid dehydrogenase 2 (HSD11B2). The activity of HSD11B1 is measured in the urine based on the (tetrahydrocortisol+5 -tetrahydrocortisol)/tetrahydrocortisone ((THF+5 -THF)/THE) ratio in humans and the (tetrahydrocorticosterone+5 -tetrahydrocorticosterone)/tetrahydrodehyd ((THB+5 -THB)/THA) ratio in mice. The cortisol/cortisone (F/E) ratio in humans and the corticosterone/11-dehydrocorticosterone (B/A) ratio in mice are markers of the activity of HSD11B2. In vitro agonist treatment of liver X receptor (LXR) down-regulates the activity of HSD11B1. Sterol 27-hydroxylase (CYP27A1) catalyses the first step in the alternative pathway of bile acid synthesis by hydroxylating cholesterol to 27-hydroxycholesterol (27-OHC). Since 27-OHC is a natural ligand for LXR, we hypothesised that CYP27A1 deficiency may up-regulate the activity of HSD11B1. In a patient with cerebrotendinous xanthomatosis carrying a loss-of-function mutation in CYP27A1, the plasma concentrations of 27-OHC were dramatically reduced (3.8 vs 90-140 ng/ml in healthy controls) and the urinary ratios of (THF+5 -THF)/THE and F/E were increased, demonstrating enhanced HSD11B1 and diminished HSD11B2 activities. Similarly, in Cyp27a1 knockout (KO) mice, the plasma concentrations of 27-OHC were undetectable (<1 vs 25-120 ng/ml in Cyp27a1 WT mice). The urinary ratio of (THB+5 -THB)/THA was fourfold and that of B/A was twofold higher in KO mice than in their WT littermates. The (THB+5 -THB)/THA ratio was also significantly increased in the plasma, liver and kidney of KO mice. In the liver of these mice, the increase in the concentrations of active glucocorticoids was due to increased liver weight as a consequence of Cyp27a1 deficiency. In vitro, 27-OHC acts as an inhibitor of the activity of HSD11B1. Our studies suggest that the expression of CYP27A1 modulates the concentrations of active glucocorticoids in both humans and mice and in vitro.

DOI: https://doi.org/10.1530/JOE-13-0141

Evidence for a Role of Sterol-27 hydroxylase in Glucocorticoid Metabolism in Vivo

Isabelle Vögeli[1], Hans H Jung[2], Bernhard Dick[1], Sandra K Erickson[3], Robert Escher[4], John W. Funder[5], Felix J Frey[1] and Geneviève Escher[1]

[1] Department of Nephrology and Hypertension, University Hospital Berne, Switzerland, [2]Department of Neurology, University Hospital Zurich, Switzerland, [3]Department of Medicine, University of California, San Francisco, CA, USA [4]Department of Internal Medicine, Emmental Hospital, Switzerland, [5]Prince Henry's Institute, Clayton 3168, Victoria, Australia.

Corresponding author and reprint requests: Geneviève Escher, PhD, Department of Nephrology and Hypertension, University Hospital Berne, CH-3010 Berne, Switzerland. Tel: ++ 41 31 632 9164; Fax: ++41 31 632 244 36; E-mail: genevieve.escher@dkf.unibe.ch

Short title: CYP27A1 and glucocorticoid metabolism in vivo

Keywords: 11β-hydroxysteroid dehydrogenase 1, (tetrahydrocorticosterone + 5α-tetrahydrocorticosterone)/11-dehydrocorticosterone ratio, 27-hydroxycholesterol, *cyp27A1* knock-out mice, cerebrotinous xanthomatosis.

Word count: 4935

Abstract

Intracellular availability of glucocorticoids is regulated by the enzymes 11β-hydroxysteroid dehydrogenase 1 (11β-HSD1) and 11β-hydroxysteroid dehydrogenase 2 (11β-HSD2). 11β-HSD1 activity is measured in urine by the (tetrahydrocortisol +5α-tetrahydrocortisol)/tetrahydrocortisone ((THF+5α-THF)/THE)ratio in humans, and (tetrahydrocorticosterone+5α-tetrahydrocorticosterone)/11-dehydrocorticosterone ((THB+ 5α-THB)/THA) in mice. The cortisol/cortisone (F/E) ratio in humans and corticosterone/11-dehydrocorticosterone (B/A) in mice is a marker for 11β-HSD2 activity. *In vitro* agonist treatment of LXR downregulates 11β-HSD1.

Sterol 27-hydroxylase (CYP27A1) catalyses the first step in the alternative pathway of bile acid synthesis by hydroxylating cholesterol to 27-hydroxycholesterol (27-OHC). Since 27-OHC is a natural ligand for LXR, we hypothesized that CYP27A1 deficiency may upregulate 11β-HSD1.

In a patient with cerebrotendinous xanthomatosis carrying a loss of function mutation in *CYP27A1*, 27-OHC plasma concentrations were dramatically reduced (3.8 ng/ml vs 90-140 ng/ml in healthy controls) and the urinary ratios of (THF+5α-THF)/THE and F/E increased, demonstrating enhanced 11β-HSD1 and diminished 11β-HSD2 activities. Similarly, in *cyp27A1* knockout (ko) mice, 27-OHC plasma concentrations were undetectable (<1 ng/ml versus 25-120 ng/ml in *cyp27A1* wild type (wt) mice). Urinary ratios of (THB+5α-THB)/THA were 4-fold, and of B/A 2-fold, higher in *cyp27A1* ko than in their wt littermates. The (THB+5α-THB)/THA ratio also increased significantly in plasma, liver and kidney of *cyp27A1* ko mice. In the liver of these mice, the increase of

11β-HSD1 activity, and increased expression of protein and mRNA were due to increased liver weight as a consequence of cyp27A1 deficiency.

Our studies suggest that CYP27A1 expression modulates active glucocorticoid concentration in both humans and mice.

Introduction

Sterol 27-hydroxylase (CYP27A1) encoded by the gene *CYP27A1* is a NADPH-dependent mitochondrial enzyme expressed in many tissues which catalyses the hydroxylation of cholesterol to 27-hydroxycholesterol (27-OHC) (Rosen, et al. 1998). In the liver, CYP27A1 catalyses the first step of the alternative pathway of bile acid biosynthesis and intermediate reactions in the classic pathway initiated by CYP7A1 (Chen, et al. 2005). In extra-hepatic tissues, CYP27A1 plays a role in reverse cholesterol transport because its product 27-OHC is removed and carried to the liver where it can be converted to bile acids (Weingartner, et al. 2010). 27-OHC is a key regulator of cholesterol homeostasis. It is one of the natural ligands for LXR, which upon activation, enhances the ABCA-1 mediated cholesterol efflux (Oram and Lawn 2001) and is a negative feedback regulator of HMG-CoA reductase, the rate-limiting enzyme for cholesterol biosynthesis (Hall, et al. 2001).

In humans, the importance of CYP27A1 in sterol homeostasis is illustrated by cerebrotendinous xanthomatosis (CTX), an autosomal recessive disease caused by a loss of function mutation in the *CYP27A1* gene (Cali, et al. 1991). Clinical features of CTX include early onset cataracts, progressive neurological dysfunction, tendon xanthomas and in some but not all patients, increased incidence of premature atherosclerosis (Cali et al. 1991). *Cyp27A1* gene knockout (ko) mice have reduced bile acid elimination and pronounced hepatomegaly (Dubrac, et al. 2005; Honda, et al. 2001; Repa, et al. 2000).

In clinical practice, glucocorticoids are widely used as anti-inflammatory and immunosuppressive agents. They have a direct effect on the transcription regulation of CYP27A1 (Tang, et al. 2008). Intracellular glucocorticoid concentrations are modulated by the 11β-hydroxysteroid dehydrogenase (11β-HSD) enzymes. 11β-HSD type 1 (11β-HSD1) preferentially catalyses the conversion of inactive 11-keto-glucocorticoids (cortisone or 11-dehydrocorticosterone) into active 11β-hydroxyglucocorticoids (cortisol or corticosterone) and by this mechanism modulates cell-specific glucocorticoid action (Agarwal, et al. 1989; Edwards, et al. 1988; Escher, et al. 1997). 11β-HSD type 2 (11β-HSD2) inactivates endogenous cortisol into cortisone (Funder, et al. 1988). In mineralocorticoid target tissues, 11β-HSD2 protects mineralocorticoid receptors from excessive activation by glucocorticoids (Atanasov, et al. 2007; Mune, et al. 1995). Regulation of cortisol metabolism into tetrahydrometabolites in the liver is catalysed by the enzyme 5α-reductase (Russell and Wilson 1994).

Recently, evidence has accumulated that 11β-HSD1 activity contributes to glucocorticoid effects in the development of the metabolic syndrome (Hermanowski-Vosatka, et al. 2005). With the ultimate goal of treating and/or preventing the metabolic syndrome, an array of exogenous compounds inhibiting 11β-HSD1 has recently been synthesized (Boyle and Kowalski 2009), and the role of endogenous compounds including insulin, glucocorticoids, TNF-alpha, bile acids and their molecular mechanisms in regulating the expression and activity of 11β-HSD were reported (Ackermann, et al. 1999; Balachandran, et al. 2008; Escher et al. 1997; Ignatova, et al. 2009; Kostadinova, et al. 2005; Quattropani, et al. 2001; Williams, et al. 2000). Thus, understanding the factors

94 regulating activity of this enzyme is of potential clinical importance. Interestingly,
95 glucocorticoids themselves regulate CYP27A1 (Tang et al. 2008).

96 The LXR family of nuclear receptors (LXRs) are ligand-activated transcription factors
97 playing roles in regulation of bile acid synthesis and metabolism, macrophage
98 cholesterol efflux and lipid metabolism (Lehmann, et al. 1997; Peet, et al. 1998;
99 Schwartz, et al. 2000). A role for LXRs in endocrine regulation was proposed by Stulnig
100 (Stulnig, et al. 2002) who showed that activation of LXRs by the agonist TO901317
101 down-regulates 11β-HSD1 expression and activity in adipocytes, an observation
102 confirmed in hepatocytes (Liu, et al. 2006).

103 Based on the interplay described between LXR and 11β-HSD1 and between 27-OHC
104 and LXR, we asked whether glucocorticoid homeostasis might differ in CTX patients so
105 that 11β-HSD1 activity, estimated by urinary glucocorticoid metabolites, may be
106 increased as compared to healthy controls. To further investigate involvement of
107 CYP27A1 in glucocorticoid metabolism, we analysed plasma, urine and tissues of
108 *cyp27A1* gene KO mice in which 27-OHC is deficient (Dubrac et al. 2005; Rosen et al.
109 1998).

110

Materials and methods

Ethics Statement

The results given for the CTX patient are parameters commonly used in routine diagnosis in our institution. The agreement in the University Hospital stipulates that results may be used anonymously for scientific purposes.

Animal experimentation was approved by the Ethical Committee for Animal Experiments of the Veterinary Administration of the Canton of Berne, Switzerland.

Case Report

The phenotype of the CTX patient has been described previously (Bartholdi, et al. 2004). Neurologically, she had progressive spastic tetraparesis and neurocognitive problems. No tendon xanthomas were found but she reported chronic diarrhea. A 24h urine sample and 10 ml blood collected in EDTA tubes were obtained and levels of 27-OHC and urinary steroid metabolites measured.

Mouse colonies

Cyp27A1 heterozygous males and females (a kind gift from Sandra K Erickson, Department of Medicine, University of California, San Francisco, USA) on a C57BL/6J genetic background were used to breed *cyp27A1* wild type (WT), heterozygous (HZ) and knockout (KO) mice. Pups were genotyped at 3 weeks (for primers and PCR conditions, see (Dubrac et al. 2005) and weaned at the age of 4 weeks. Males used for the experiment were maintained on standard rodent chow. Urine was collected from 4 to 6 month old

134 animals as follows. Mice were housed separately for one week, placed for pre-adaptation
135 in a metabolic cage for 3 days and urine collected every 24h for the next 3 days. At the
136 end of the collection, mice were starved for 12 hours, sacrificed by pentobarbital injection
137 (300 mg/kg, pentobarbital sodium, USP, Abbott Laboratories North Chicago, IL60064,
138 USA), weighed and blood collected into a tube containing 20-50 units heparin. Plasma
139 was centrifuged and stored at –20°C until use. Organs were removed, weighed, frozen in
140 liquid nitrogen and stored at -80°C until use.
141
142 **Plasma and liver biochemistry**
143 Glucose, cholesterol, triglycerides, ALT and AST were measured by
144 electrochemiluminescence immunoassay (Roche Diagnostics, Switzerland). Insulin was
145 measured with the ultra sensitive rat/mouse insulin Elisa kit (Crystal Chem Inc (Cat No
146 90060), Downers Grove, IL, USA). 27-OHC was quantified by GC-MS as previously
147 described (Burkard, et al. 2004) with 100 ng 5α-cholestan-3β,6α-diol and 100 ng
148 stigmasterol as standards; for details, see supplementary material.
149 Triglyceride content was measured in liver homogenates with a triglyceride
150 Quantification Kit (BioVision (Cat No K622-100), Mountain View, CA, USA).
151
152 **Analysis of steroid metabolites by gas chromatography-mass spectrometry**
153 For both mouse and human samples, urinary steroids were extracted from 1.5 ml urine
154 and analysed by GC-MS as previously described (Ackermann et al. 1999; Escher, et al.
155 2009; Odermatt, et al. 2001). In human urine, tetrahydrocortisol (THF), 5α-
156 tetrahydrocortisol (5α-THF), tetrahydrocortisone (THE), cortisol (F), cortisone (E), α-

157 cortol, β-cortol, α-cortolone, β-cortolone were quantified. In mouse urine
158 tetrahydrocorticosterone (THB), 5α-tetrahydrocorticosterone (5α-THB), 11-dehydro-
159 tetrahydrocorticosterone (THA), corticosterone (B) and dehydrocorticosterone (A) were
160 measured. Glucocorticoid metabolites were also quantified in plasma (100-400 µl), liver
161 and kidney tissues (100 mg). Following an extraction step with10 vol dichloromethane,
162 steroids were extracted in the organic phase and derivatized. The detection limit was 1
163 ng/ml for plasma or urine and 1 ng/100 mg for tissue.
164
165 **Enzymatic assays**
166 **11β-HSD1 activity in mouse liver homogenates**
167 Reduction of cortisone to cortisol was used to assess 11β-HSD1 activity. Frozen liver
168 samples were powdered and 30-50 mg tissue homogenized with 500 µl buffer containing
169 250 mM sucrose, 10 mM Tris Base pH 7.5 and 1 mM PMSF. Protein was quantified with
170 a BCA^{TM} Protein Assay Kit (Pierce Cat No 23225, Rockford, IL 61105, USA). For 11β-
171 HSD1 assay, 10 µg protein was incubated for 30 min at 37°C in the presence of 951 mol/l
172 β-nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt (NADPH),
173 1.5 µmol/l cortisone and 3.2 nCi of [^{3}H]-cortisone in sucrose buffer. The reaction was
174 stopped by adding 30 µl of a mixture containing 10 mg/ml cortisone and cortisol in
175 methanol. Samples were spotted onto TLC plates ((G-25, UV254) Macherey-Nagel,
176 Oensingen, Switzerland) and developed in a solvent containing chloroform-methanol
177 (90:10 v/v). Steroids were visualized under ultraviolet light, the spots removed and
178 counted in a Packard scintillation counter (Tri-Carb 2000CA; United Technologies,
179 Hartford, CT, USA), and the percentage of conversion of cortisone to cortisol calculated.

11β-HSD2 activity in kidney homogenates

11β-HSD2 activity was assessed by measuring oxidation of corticosterone to dehydrocorticosterone. Kidney homogenates were prepared as described above for liver from 30-50 mg of frozen tissue. Assay of 11β-HSD2 activity was performed using 10 μg protein and incubated for 90 min at 37°C in a mixture containing 200 mol/l nicotinamide adenine dinucleotide (NAD), 10 nmol/l corticosterone and 3.2 nCi of [^{3}H]-corticosterone in sucrose buffer. The reaction was stopped by adding 30 μl of a mixture containing 10 mg/ml corticosterone and dehydrocorticosterone in methanol. TLC was performed as described above and the percentage of conversion of corticosterone to dehydrocorticosterone calculated.

RNA extraction and real-time PCR

Total RNA was isolated from frozen tissues with a SV Total RNA Isolation System Kit (Promega Cat No Z3100, Madison, WI, USA). Reverse transcription was performed with 2 μg RNA in a reaction containing 100 Units SuperScript Reverse Transcriptase type II according to the manufacturer's protocol (Invitrogen Cat No 18064-022). Real-time PCR was performed with specific TaqMan Gene Expression Assays and 18S or β-actin as the housekeeping gene, using ABI Prism 7500 Fast Sequence Detection System (version 1.4).

Western Blot analysis

20 μg of liver homogenates was separated on a 12.5% SDS polyacrylamide gel by electrophoresis, followed by immunoblotting with anti-11β-HSD1 rabbit antiserum (kind

gift from C. Monder) at a dilution of 1:1'000 in 2% non-fat dried milk. Bands were visualized with an Enhanced Chemiluminescence kit (Amersham Cat No RPN2106) and quantified by densitometry using Image J. For internal standard, β-actin (Santa-Cruz) was used at a dilution of 1:3'000 in 2% non-fat dried milk.

Effect of 27-OHC on 11β-HSD1 activity in vitro

For dose-dependent inhibition studies, CHOP cells were transfected as described previously (Escher et al. 2009) with a plasmid encoding 11β-HSD1. Two days following transfection, cells were harvested, homogenized in sucrose buffer (100 mM sucrose, 10 mM Tris pH 7.4) and enzymatic assays and TLC were performed as described above in the presence of increasing concentration of 27-OHC diluted in ethanol.

To measure the direct effect of CYP27A1 expression on 11β-HSD1 activity, CHOP cells were co-transfected with 2 plasmids, one encoding 11β-HSD1 and the other CYP27A1 or pcDNA3. Enzymatic assay was performed for 4h at 37°C and protein amount was determined to calculate the specific activity.

Statistical analysis

To determine significant differences, one-way ANOVA was used, followed by Bonferroni post-hoc tests for multiple comparisons or Kruskal-Wallis followed by Dunn's multiple comparison tests.

Results

Analysis of 27-OHC and glucocorticoid metabolites in the patient with CTX

The concentration of 27-OHC in plasma from the CTX patient was reduced (3.8 ng/ml versus 90-140 ng/ml normal range in controls) (Burkard et al. 2004), reflecting decreased sterol 27-hydroxylase activity (Table 1). To establish whether this decreased concentration of 27-OHC was mirrored by changes in glucocorticoid ratios reflecting 11β-HSD1 activity, metabolites of glucocorticoids were determined in plasma and urine by GC-MS. In plasma, F was in the normal range when compared to controls obtained from a previous study (N'Gankam, et al. 2002) but no E was detected (Table 1). In urine, increased (THF+5α-THF)/THE and cortols/cortolones, indicating increased reductive activity of 11β-HSD1, and an increased F/E ratio reflecting diminished oxidative activity of 11β-HSD2 were observed. The ratio THF/5α-THF, a measure of 5α-reductase activity, remained unchanged (Table 1).

Phenotype changes in *cyp27A1* deficient mice

Body weight remained unchanged but liver size was almost twice as large in *cyp27A1* ko mice as in wt or hz mice (Table 2). Kidneys, spleen, lungs, testis and brain weight were unchanged (data not shown). Plasma 27-OHC was undetectable in *cyp27A1* ko mice but not significantly reduced in *cyp27A1* hz mice (Table 2). CYP27A1 deficiency led to an increase of plasma ALT and AST plus a decrease of cholesterol, a lower glucose and higher insulin whereas plasma triglyceride levels were unchanged (Table 2) despite accumulation of triglycerides in *cyp27A1* ko livers (Table 2).

Analysis of glucocorticoid metabolites in *cyp27A1* ko mice

11β-HSD1 reductive activity assessed from the ratio (THB+5α-THB)/THA increased 4-fold in urines of *cyp27A1* ko mice compared with wt or hz mice (Fig. 1A). The B/A ratio ratio increased more than 2 fold, indicating an apparent decrease of 11β-HSD2 oxidative activity (Fig. 1B). The THB/5α-THB ratio, a marker for 5α- reductase activity, was markedly reduced in *cyp27A1* ko mice, indicating enhanced A-ring reduction of THB (Fig. 1C). The total amount of steroids excreted in 24h was unchanged (data not shown) and the (THB+5α-THB)/THA ratio correlated positively with liver size (Fig. 1D). The (THB+5α-THB)/THA ratio was also significantly increased in plasma, liver and kidney of *cyp27A1* ko mice (Fig 2A, C and D) with no changes observed in adrenals (data not shown). The B/A ratio also increased in plasma of *cyp27A1* ko mice (Fig. 2B) and similarly was unchanged in adrenals (data not shown). Both B and A were below the detection limit in liver and kidney tissue.

Assessment of 11β-HSD1 activity, protein and mRNA levels in mouse livers and kidneys

11β-HSD1 activity was measured in liver homogenates, with levels of both shown to be independent of *CYP27A1* genotype (Fig. 3A and 3B). 11β-HSD1 protein content was quantified by Western Blot in liver homogenates, with β-actin as internal control (Fig. 3C). The amount of 11β-HSD1 protein correlated with liver size (Fig. 3D).

In liver tissue, 11β-HSD1 mRNA levels were similar across all 3 genotypes (Fig. 4A). The mRNA level of hexose-6-phosphate dehydrogenase (H6PDH), a known modulator of 11β-HSD1 activity (Bujalska, et al. 2005; Walker, et al. 2007), was similarly unchanged (Fig. 4B), as were 11β-HSD1and 11β-HSD2 mRNA in kidney tissue from all 3 genotypes (Fig. 4C and 4D).

Inhibition of 11β-HSD1 by 27-OHC in vitro

The effect of 27-OHC on 11β-HSD1 activity was assessed in vitro in a lysate of CHOP cells transfected with a plasmid encoding 11β-HSD1.27-OHC inhibited 11β-HSD1 in a dose-dependent manner (Fig. 5A). Similarly, 11β-HSD1 specific activity was significantly reduced in CHOP cells transfected with 11β-HSD1 when CYP27A1 was expressed (Fig. 5B).

Discussion

Regulation of 11β-HSD1 and 11β-HSD2 enzymes by different endogenous molecules and xenobiotics and in various disease states has been previously described and appears to be clinically relevant (Escher et al. 1997; Escher, et al. 1998a; Escher, et al. 1998b; Fuster, et al. 1998; Heiniger, et al. 2003; Ignatova et al. 2009; Konopelska, et al. 2009; Kostadinova et al. 2005; Quattropani et al. 2001). Here we add a novel player, 27-OHC, regulating the activity of these key enzymes determining intracellular glucocorticoid availability. As a confirmation of our hypothesis, and schematised in Fig. 6, we showed a diminished production of 27-OHC in the CTX patient, and diminished 27-OHC enhanced LXR-driven inhibition of 11β-HSD1. As a result, the conversion of inactive to active glucocorticoids increased reflecting increased activity of 11β-HSD1, as shown by the absence of E in plasma and elevated urinary (THF+5α-THF)/THE and cortol/cortolone ratios. Combined with an increased F/E ratio, an apparent indicator of reduced 11β-HSD2 activity, an overall increase of active glucocorticoids was found in the urine of the CTX patient analysed.

Similarly, in the absence of circulating 27-OHC, *cyp27A1* ko mice have an increased (THB+5α-THB)/THA ratio in urine but also in plasma, liver and kidney. With an increased B/A ratio in plasma and urine, *cyp27A1* ko mice tissues are clearly exposed to increased levels of active glucocorticoids.

In vitro, 27-OHC inhibited 11β –HSD1 activity in a dose-dependent manner, and over-expression of CYP27A1 led to reduced 11β-HSD1 activity (Fig. 5). Thus 27-OHC itself acts as inhibitor on11β-HSD1 (Fig. 6).

Similar changes observed in the steroid ratios occur as a consequence of a reduced bile acid production (Cali et al. 1991; Dubrac et al. 2005; Peet et al. 1998; Rosen et al. 1998). It has been shown that removal of a biliary obstruction in patients with cholestasis with a subsequent decrease in the concentrations of bile acids in plasma had similar impacts on the urinary ratios of (THF + 5αTHF)/THE (Quattropani et al. 2001). CTX patients including the present patient are classically treated with chenodeoxycholic acid (CDCA), a substance known to inhibit 11β-HSD1 and 11β-HSD2 (Ackermann et al. 1999). This treatment could explain the different level of increase in the urinary ratio between the CTX patient and the *cyp27A1* ko mice. Without treatment with bile acids, *cyp27A1* ko mice had a 4 fold increase in the (THB+5α-THB)/THA ratio, whereas (THF+5α-THF)/THE was only modestly increased in our patient who received CDCA.

Besides 11β-HSD1 activity, the total levels of glucocorticoids excreted and the enzyme 5α-reductase are also important determinants of the F/E and (THF+5α-THF)/THE ratios. In this study, the amount of steroids excreted in urine was in the same range for the 3 groups of mice, and in the CTX patient within control values (data not shown). By contrast, 5α-reductase activity was unchanged in the CTX patient but decreased in *cyp27A1* ko mice (Table 1 & Fig. 1C), a finding that may reflect a difference between humans and mice as to the phenotypic expression of CYP27A1 depletion, i.e. hepatic steatosis in *cyp27A1* ko mice and its absence in the CTX patient. A recent study (Ahmed, et al. 2012) showed an increased 5α-THF/THF ratio in patients with steatosis compared to controls, a finding in line with the reduction of THB/5α-THB in *cyp27A1* ko mice.

The role of endogenous glucocorticoids in the pathogenesis of the metabolic syndrome and individuals suffering from the Cushing's syndrome is well established, as well as

their involvement in hepatic triglyceride accumulation (Dourakis, et al. 2002). In non-alcoholic fatty liver disease, one of the manifestations of the metabolic syndrome, increased clearance and decreased hepatic regeneration of cortisol has been proposed as a protective mechanism to decrease local glucocorticoid availability (Ahmed et al. 2012). The clinical implication of the increased glucocorticoid availability in our CTX could be of interest. Given the glucocorticoid responsive element in the promoter sequence of CYP27A1, increased cortisol concentration might contribute substantially to CYP27A1 residual activity. Even more, this could explain the different susceptibility of CTX to develop or not atherosclerosis.

In the present study, we found that *cyp27A1* ko mice have increased circulating and tissue glucocorticoid levels (Fig. 2~~A~~) that could, at least in part, explain their hepatomegaly. Taken together, our results indicate that the reduction of 27-OHC production by CYP27A1 and its effect on 11β-HSD1 activity enhances tissue glucocorticoid concentration and could, at least in part, lead to the development of steatosis. Administration of 27-OHC to LDL-receptor ko mice has been shown to reduce the accumulation of lysosomal cholesterol and hepatic inflammation in the liver (Bieghs, et al. 2013). If reduced 27-OHC concentrations are found in the circulation of patients with fatty liver, enhancing CYP27A1 activity might then be a viable therapeutic option.

The changes in glucocorticoid ratios observed in *cyp27A1* ko mice were not regulated at the level of 11β-HSD1 mRNA or protein in the liver (Fig. 3C & 4), and the specific activities of 11β-HSD1 and 11β-HS2 were similarly not changed (Fig. 3A &B). Thus, we might speculate that the increased (THB+5α-THB)/THA ratio observed in urine, plasma and liver are solely due to (i) increased liver mass, (ii) increased hepatic capacity to

351 regenerate active glucocorticoids via 11β-HSD1, (iii) decreased bile acid production, (iv)
352 cofactor availability, and~~/or~~ (v) to direct or indirect inhibition of 11β-HSD1 via
353 activation of LXR by 27-OHC.
354 In conclusion, we show that reduced CYP27A1 activity enhances active glucocorticoid
355 levels in humans and mice. It is the first evidence that not only xenobiotics but enzymatic
356 activity are involved in the regulation of 11β-HSD1. In addition, hepatomegaly and
357 hepatic steatosis in *cyp27A1* ko mice may also be a consequence of increased
358 glucocorticoid availability.
359

Declaration of interest

The authors have no conflicts of interest to declare.

Funding

This work was supported by two grants from the Swiss National Foundation to Geneviève Escher (310000-109774 and 310030-122133).

Author contributions

IV, BD and GE contributed to the production of the results. HHJ organised the CTX patient and SKE provided the cyp27A1 mouse colony. SKE, RE, JWF, FF and GE participated in the redaction of the manuscript.

Acknowledgments

We thank Beatrice Blanchard and Pamela Hayoz for technical assistance.

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Figure legends

Figure 1. Effect of CYP27A1 deficiency on the excretion of urinary glucocorticoid metabolites in mice.

Steroids were extracted from *cyp27A1* wt, hz and ko mice urines and analysed by GC-MS. The (THB+5α-THB)/THA ratio increased ~4 fold in *cyp27A1* ko mice compared to wt, indicating an increased apparent 11β-HSD1 reductive activity (A). The B/A ratio (B) doubled in *cyp27A1* ko mice, indicating reduced apparent 11β-HSD2 oxidase activity. The THB/5α-THB ratio, a measurement of liver 5α-reductase, fell ~ 6 fold in *cyp27A1* ko mice (C). There was a correlation between the (THB+5α-THB)/THA ratio and liver size ($p= 0.0003$) (circles represent *cyp27A1* wt, squares *cyp27A1* hz and triangles *cyp27A1* ko mice) (D). Mouse urine sample number : n=9, 7, 6 for *cyp27A1* wt, hz and ko mice, respectively. Means with whiskers (minimum and maximum) are represented. One-way ANOVA followed by Bonferroni post-hoc tests for multiple comparisons were used to test statistical differences, with *** $p<0.0001$ and ** $p<0.001$for *cyp27A1* ko versus wt mice.

Figure 2. Effect of CYP27A1 deficiency on plasma and tissue glucocorticoid metabolites in mice.

Steroids were extracted from plasma, liver and kidney and analysed by GC-MS. The (THB+5α-THB)/THA and B/A ratios increased by ~50% in plasma (A and B). The ratio (THB+5α-THB)/THA increased by less than 50% in liver (C) and kidney (D) homogenates. Number of mouse samples: n=5; means with whiskers (minimum and

maximum) are represented. One-way ANOVA followed by Bonferroni post-hoc tests for multiple comparisons or Kruskal-Wallist tests followed by Dunn tests were used to test statistical differences, with *** $p<0.0001$ and ** $p<0.001$ for *cyp27A1* ko versus wt mice.

Figure 3. Effect of CYP27A1 deficiency on 11β-HSD1 and 11β-HSD2 activity in mouse liver and kidney and 11β-HSD1 protein in liver.

Activity was assayed in liver (11β-HSD1) (A) and kidney (11β-HSD2) (B) homogenates from *cyp27A1* wt, hz and ko mice and expressed as percentage of conversion of substrate to product. No difference between the 3 groups of animals was seen. 11β-HSD1 protein was quantified in liver homogenates by Western Blot, with β-actin as internal standard. A representative blot is shown (C) with lane 1, *cyp27A1* wt; lane 2, *cyp27A1* hz; lane 3, *cyp27A1* ko. The relative amount of 11β-HSD1 protein increased with liver size ($p=0.0387$) (D). Number of samples: n=8, 10 and 7 for *cyp27A1* wt, hz and ko mice, respectively, and values are represented as means and whiskers (minimum and maximum). Circles represent *cyp27A1* wt, squares *cyp27A1* hz and triangles *cyp27A1* ko mice.

Figure 4. Quantification of hepatic and renal 11β-HSD1, 11β-HSD2 and H6PD mRNA.

In liver, there was no change of 11β-HSD1 (A) and H6PDH (B) mRNA in *cyp27A1* ko mice as compared to *cyp27A1* wt littermates; there was similarly no change in 11β-HSD2 (C) or 11β-HSD1 (D) mRNA in kidney. Values are represented as means with whiskers (minimum and maximum); n=7, 13 and 10 for *cyp27A1* wt, hz and ko mice, respectively.

Figure 5. Effect of CYP27A1 expression on 11β-HSD1 activity in vitro.

CHOP cells were transfected as described in the method section. (A) 27-OHC inhibited 11β-HSD1activity in a dose dependently. (B) Specific activity was reduced in CHOP cells overexpressing 11β-HSD1 and transfected with CYP27A1 when compared to those transfected with p. The figures are representative experiments performed in triplicates. Values are mean ± SD.

Figure 6. Tissue specific interplay between CYP27A1 and glucocorticoid metabolism.

Cortisol (F), cortisone (E) and their corresponding metabolites 5α-tetrahydrocortisol (5α-THF), 5β-tetrahydrocortisol (THF) and tetraydrocortisone (THE), and in addition cortols and cortolones can be quantified in urine and used as markers for local regulation of 11β-HSD1 and 11β-HSD2 enzyme activity. In liver, conversion of cortisone to cortisol by 11β-HSD1 is inhibited by both 27-OHC and bile acids. 27-OHC inhibits 11β-HSD1 indirectly via LXR (Stulnig et al. 2002) and directly (Fig. 5). Thus, reduced CYP27A1 activity causes an increased activity of 11β-HSD1, an overall effect favouring the formation of cortisol and its metabolites. Furthermore, the formation of cortisol in tissues expressing 11β-HSD2, mainly the kidney, is enhanced since bile acids inhibit 11β-HSD2 activity, i.e. the conversion of cortisol into cortisone. Thus, overall absence of CYP27A1 favours the formation of cortisol and its metabolites.

Figure 1

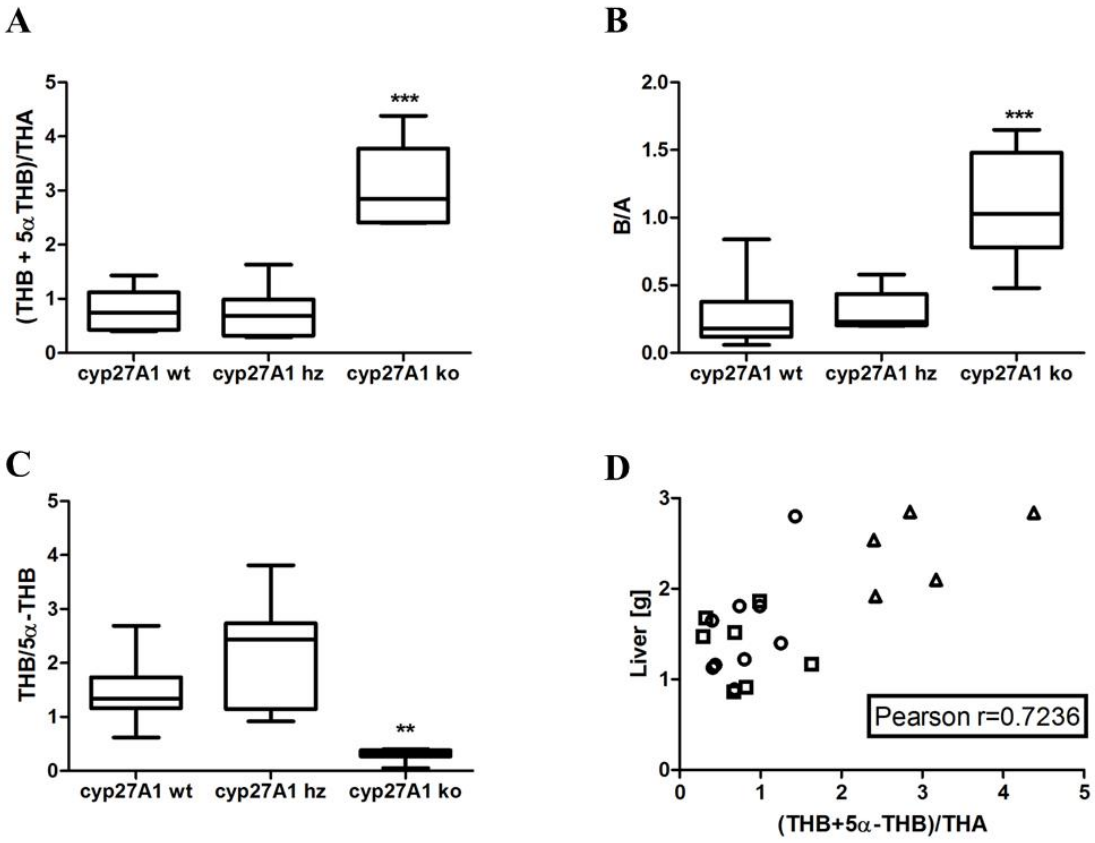
A
(THB + 5α THB)/THA
***
cyp27A1 wt
cyp27A1 hz
cyp27A1 ko
B
B/A
***
cyp27A1 wt
cyp27A1 hz
cyp27A1 ko
C
THB/5α-THB
**
cyp27A1 wt
cyp27A1 hz
cyp27A1 ko
D
Liver [g]
Pearson r=0.7236
(THB+5α-THB)/THA

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Figure 2

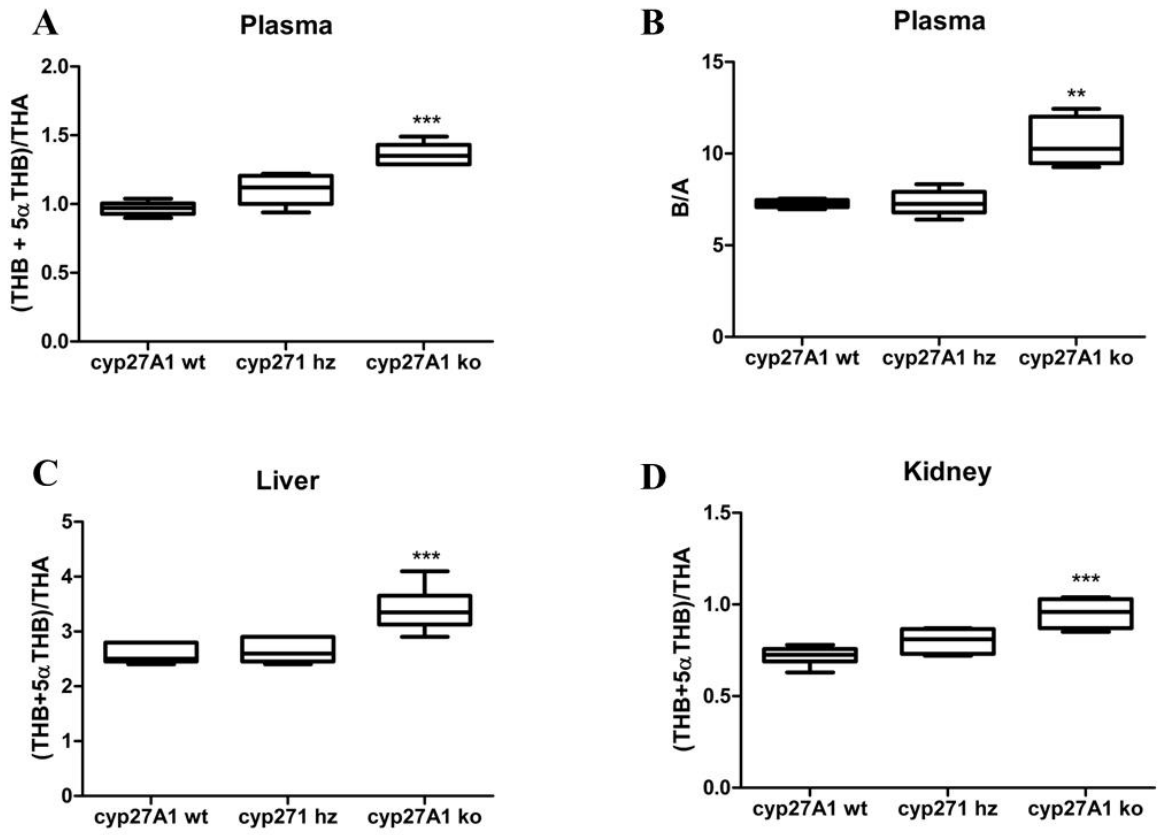

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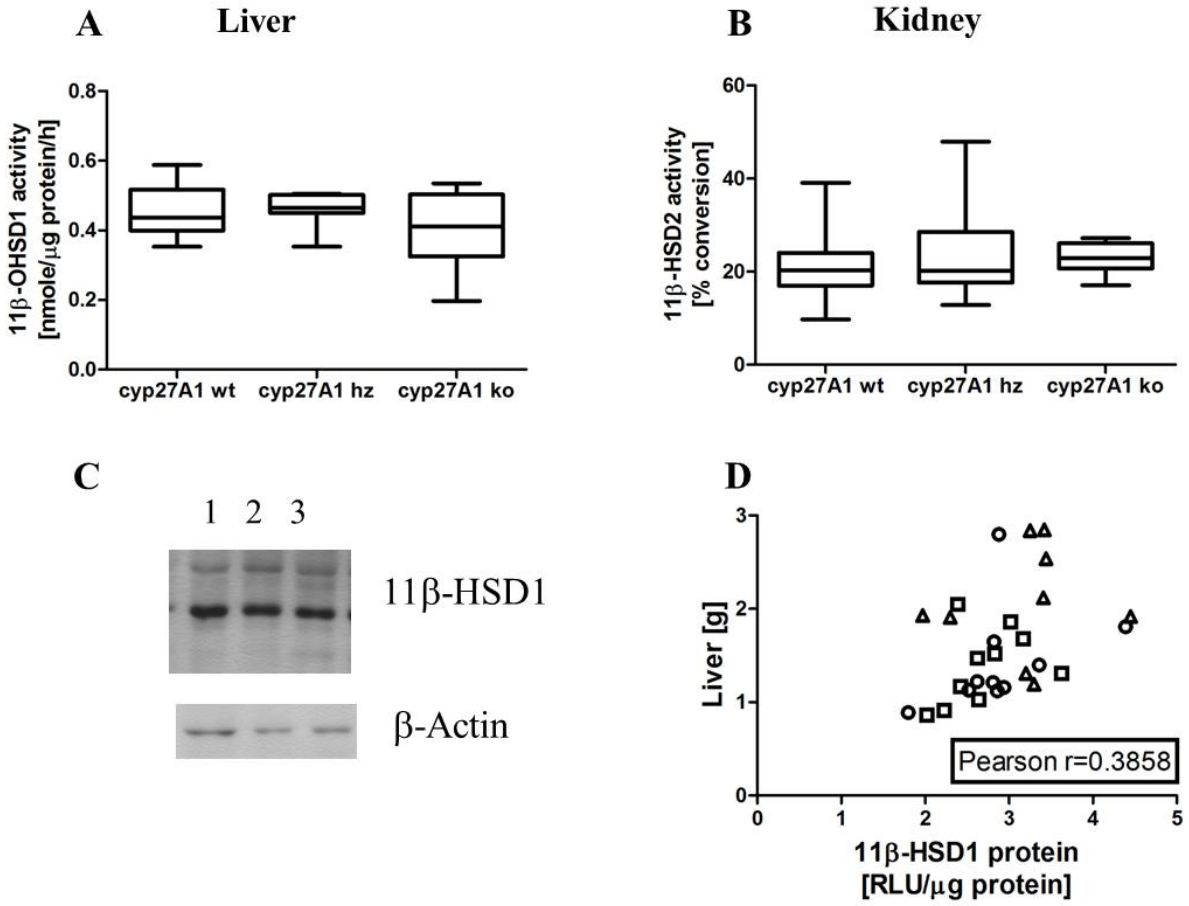
Figure 3



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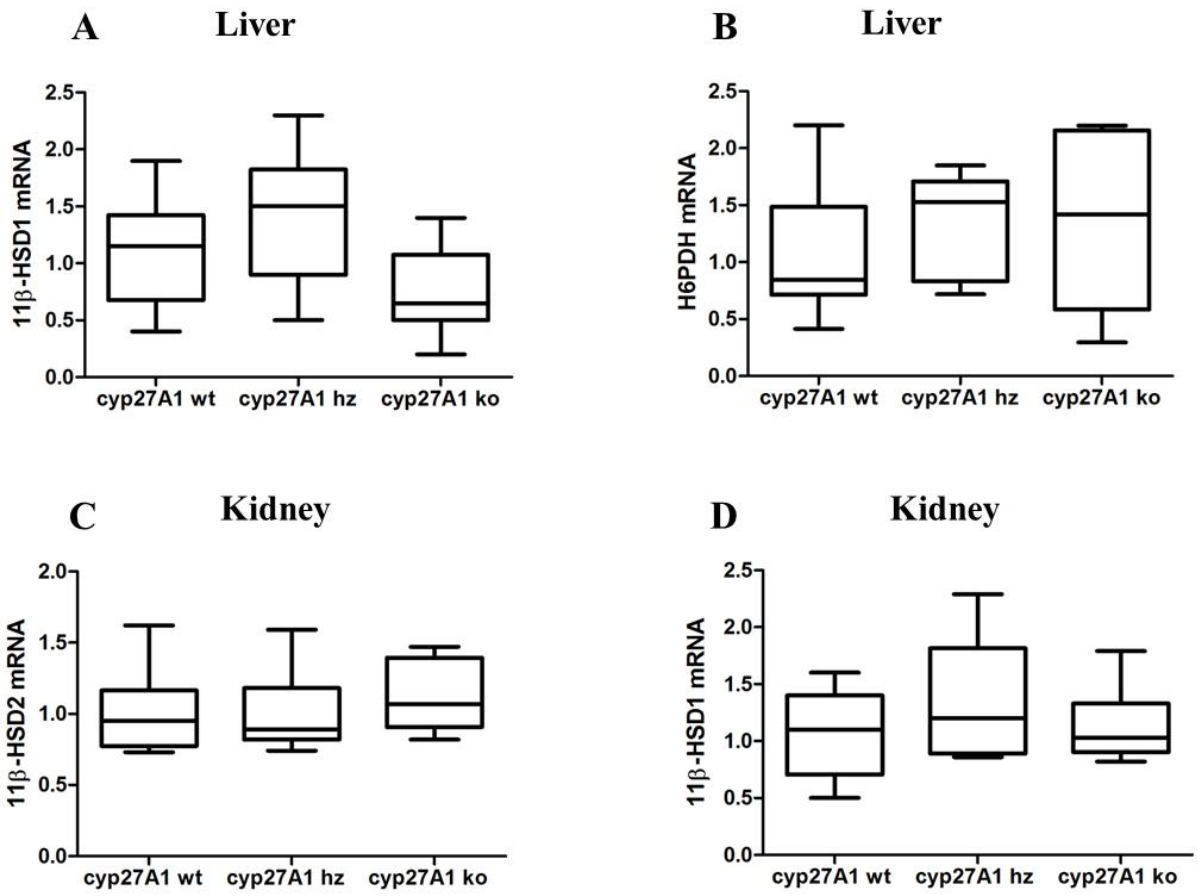
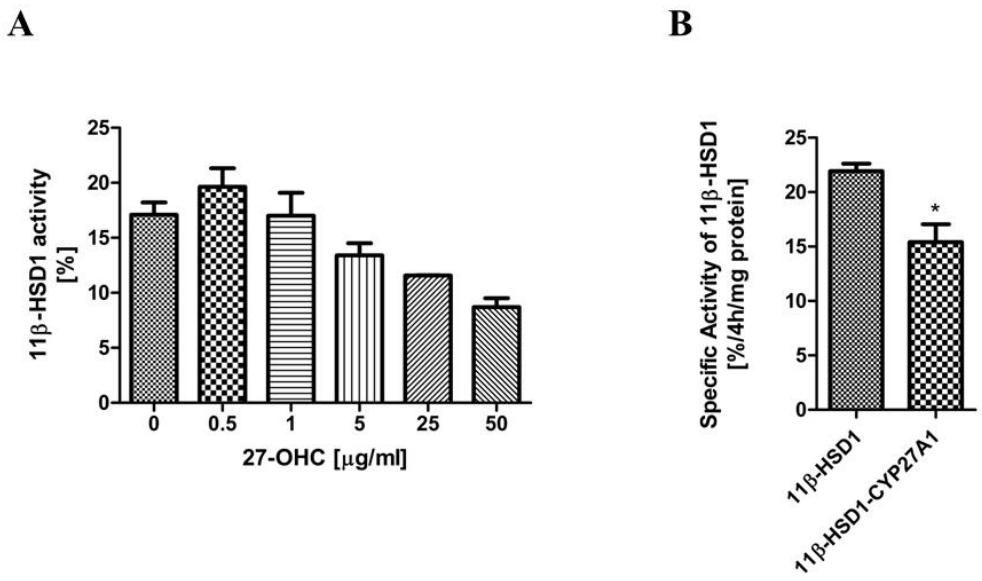

A
Liver
11β-HSD1 mRNA
2.5
2.0
1.5
1.0
0.5
0.0
cyp27A1 wt
cyp27A1 hz
cyp27A1 ko
B
Liver
H6PDH mRNA
2.5
2.0
1.5
1.0
0.5
0.0
cyp27A1 wt
cyp27A1 hz
cyp27A1 ko
C
Kidney
11β-HSD2 mRNA
2.0
1.5
1.0
0.5
0.0
cyp27A1 wt
cyp27A1 hz
cyp27A1 ko
D
Kidney
11β-HSD1 mRNA
2.5
2.0
1.5
1.0
0.5
0.0
cyp27A1 wt
cyp27A1 hz
cyp27A1 ko

593
594

Figure 5



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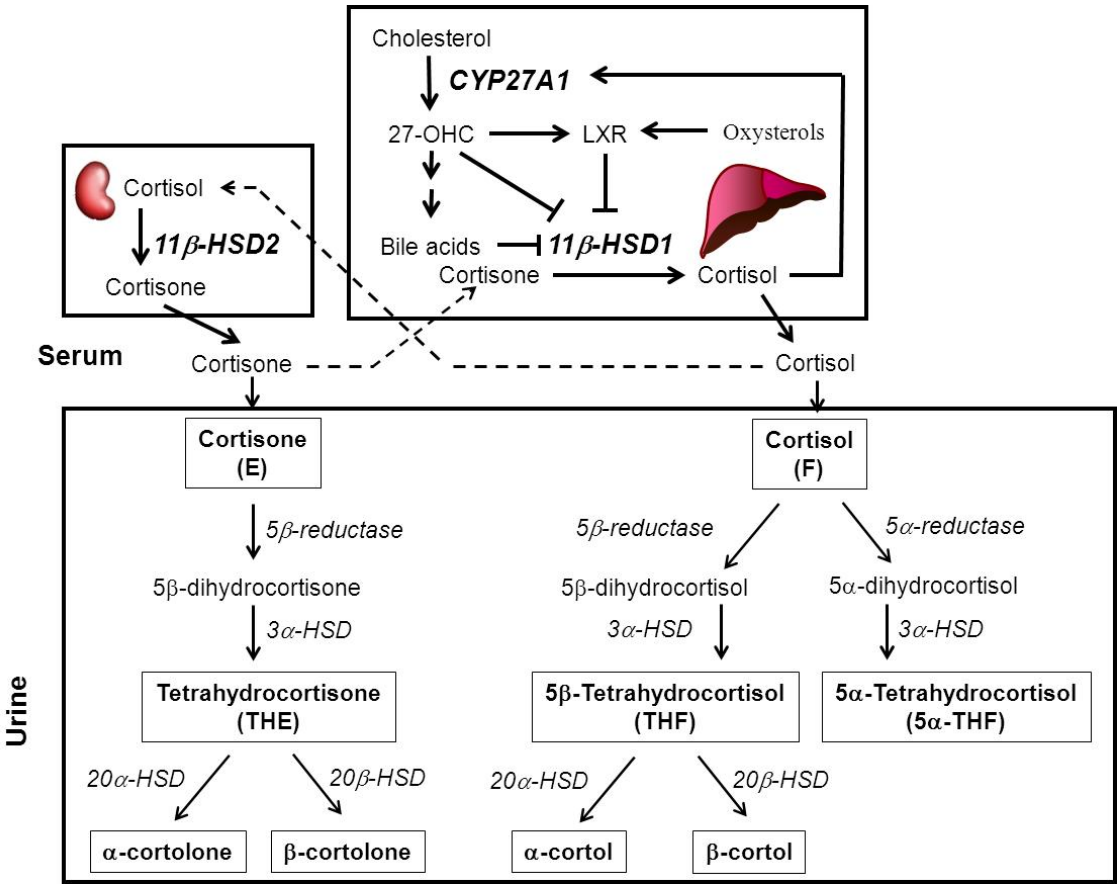
Cholesterol
CYP27A1
27-OHC
LXR
Oxysterols
Cortisol
11β-HSD2
Cortisone
Bile acids
11β-HSD1
Cortisone
Cortisol
Serum
Cortisone
Cortisol
Urine
Cortisone (E)
Cortisol (F)
5β-reductase
5β-reductase
5α-reductase
5β-dihydrocortisone
5β-dihydrocortisol
5α-dihydrocortisol
3α-HSD
3α-HSD
3α-HSD
Tetrahydrocortisone (THE)
5β-Tetrahydrocortisol (THF)
5α-Tetrahydrocortisol (5α-THF)
20α-HSD
20β-HSD
20α-HSD
20β-HSD
α-cortolone
β-cortolone
α-cortol
β-cortol

597